PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| Applicants | : | Richard Ian Christopherson et al. |
| Application No. | : | 09/888,959 |
| Filed | : | June 25, 2001 |
| For | : | AN ASSAY TO DETECT A BINDING PARTNER |

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| Examiner | : | Anne L Holleran |
| Art Unit | : | 1642 |
| Docket No. | : | 650061.401C1 |
| Date | : | September 13, 2004 |

**DECLARATION UNDER 37 C.F.R. § 1.132**
**OF RICHARD IAN CHRISTOPHERSON**

Commissioner for Patents
Washington, D.C. 20231

I, Richard Ian Christopherson, hereby declare:

1. I am currently Professor in the School of Molecular and Microbial Biosciences at the University of Sydney, Maze Crescent, Sydney, New South Wales, 2006, Australia. My Curriculum Vitae attached hereto as Exhibit A, which includes a listing of publications that I have authored or co-authored and which evidence my expertise *inter alia* in flow cytometric technology and cancer diagnosis.

2. I am a co-inventor of U.S. Patent Application No. 09/888,959 (hereinafter referred to as "USSN '959"). The claimed subject matter relates to an assay for identifying a type of leukemia by contacting a biological sample from a patient with an array of immunoglobulin molecules bound to a solid support. The assay steps require the

interaction of antigens present on the surface of the cell in the biological sample with those immunoglobulin molecules bound to the solid support. A diagnosis is then performed by establishing a pattern of interaction that is a differential pattern of density providing an identifiable signal. The differential pattern identified is determinative of either the absence of leukemia or the presence of a specific type of leukemia.

3. The ability to distinguish between a multitude of different leukemias requires the examination of multiple surface markers. For example, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HCL), B cell lymphoma (BCL), and acute myeloid leukemia (AML) may be distinguished using a panel of antibodies specific for different cell surface antigens (*see* USSN '959, page 64, lines 9-14; Table 7). This analysis is possible due to the nature of the claimed method, which allows for the concurrent detection and comparison of at least seven cell surface antigens, as many as about fifty cell surface antigens (*see, e.g.*, page 44, lines 24-30; Tables 1-7); and as many as about 1000 antigens (*see, e.g.*, page 35, lines 9-18) concurrently within a single assay.

4. I have reviewed the Office Action dated June 3, 2004, and the documents cited therein in the rejection under 35 U.S.C. § 103 has been explained to me by patent advisors. The Action alleges that the presently claimed method would have been obvious to a person having ordinary skill in the art over Chang (U.S. Patent No. 4,591,570) in light of Valet et al. (*Cytometry* 30:275-288, 1997); Terstappen et al. (U.S. Patent No. 5,234,816); Verwer et al. (U.S. Patent No. 5,605,805); or Orfao de Matos Correira E Val (U.S. Patent No. 5,538,855). The Action also alleges that the claimed method would have been obvious to a person having ordinary skill in the art over Hoeffler (U.S. Publication No. 2002/0164656) in view of Terstappen (U.S. Patent No. 5,234,816).

5. I submit, as a highly skilled artisan, that a person having ordinary skill in the art would not have been motivated to combine the teachings of Chang with any of the cited documents according to the teachings therein to achieve the claimed method with an expectation of success. Chang (U.S. Patent No. 4,591,570) can be contextualized by

reference to Chang (*J. Immunol. Meth.* 65 (1983) 217-223). These references merely describe a method using three antibodies immobilized on a solid support for the purpose of determining the proportion of specific subsets of B-cells, T-cells and monocytes in the mononuclear cell fraction. One type of antibody was used to measure each of these subsets, there was no use or mention of patterns of recognition, protein expression profiling or disease signatures. Moreover, as a skilled artisan, I submit that I would not have found that Chang motivated me in any way to use an array of Chang's design to identify or monitor *any* type of disease state. Chang also does not mention or suggest that binding of an array of immunoglobulins to different cell surface antigens results in any pattern whatsoever such as a pattern of interaction that is a differential pattern of density that provides an identifiable signal. The differential pattern of interaction indicates the relative density of interaction between each immunoglobulin and its cognate cell surface antigen, which may result, for example, from differential density of cells that bind to a discrete spot on the array, the differential expression of particular antigens, and/or the number of antigens per cell (*see, e.g.,* specification, page 26, lines 11-20). The identifiable signal therefore may vary not only with a particular cell type such as a different type of leukemia but may vary with the level of expression of a particular antigen on different cell types, which results in a pattern of interaction that may be visualized and/or quantified *(see, e.g.,* specification, Tables 1-8). Chang is completely silent on this critical aspect that provides a unique identifiable signal, likely because it was as unknown to him as others of skill in the art at the time of filing the present application.

Further, we do not consider that the methods of Chang can be reproduced or practiced by one of skill in the relevant art. Specifically, we have replicated the experiments described in working examples of Chang and have found that the data obtained are not reliable. We have found, like others, that antibodies adhered directly to glass do not reliably remain adhered to the surface of the glass unless the surface is first derivatised. The derivatisation of glass surfaces to anchor ligands is technology which was not available at the time of filing of USSN '959 and has only subsequently been

developed. Accordingly, when using the assay device described in Chang to determine the presence of specific antigens on a cell surface, one would not be able to differentiate between a cell population which is negative for the surface antigen(s) being tested versus a negative result due to the loss of antibodies from the surface of the glass slide during cell capture and subsequent washing. Similarly, it is difficult to block acid-washed glass with a protein solution such as skim milk.

6. To demonstrate the unsatisfactory outcome of the experiments disclosed in Chang (1983, 1986) the following experiments were performed. The experiment was run on 29 June 1999 by Ms Odetta de la Vega and Dr Larissa Belov. Briefly, the method of Chang (1983, 1986) was tested in the following experiment. The slides used were Oncyte slides with a nitrocellulose film from Molecular Probes (Eugene OR, manufactured by Grace Biolabs, Bend OR), silanated (amine) slides from Telechem International (Sunnyvale, CA) and plain glass microscope slides (acid washed). Antibody dots (200 µL, 200 µg/mL) were applied to the 3 types of glass slides and dried for 60 minutes. The slides were immersed in phosphate-buffered saline (PBS) and then in skim milk (5% w/v Diploma, Melbourne, Australia) for 15 min at 37°C. The slides were dried for 60 min at room temperature, and then stored dry at 4°C.

Mononuclear leukocytes were prepared from 50 ml of normal blood by centrifugation (1,600 rpm, 600 g, 30 min, room temperature) on 30 mL of Histopaque. Mononuclear leukocytes were collected at the interface, washed twice in Hanks solution and resuspended in Hanks at a density of $10^7$ cells/mL. The slides with antibody dots were moistened in PBS and then 500 µL of the leukocyte suspension (normal peripheral blood leukocytes) was placed on the array on the slide and incubated at 37°C for 30 min. The slides were gently washed in PBS to remove unbound cells and observed microscopically. The Oncyte slides gave a well defined dot pattern for normal peripheral blood leukocytes (mononuclear fraction) which was photographed using 25-fold magnification with Nomaski optics. There was no result from the Telechem or plain glass slides due to high background binding of leukocytes to the glass between the

antibody dots. The nitrocellulose film of the Oncyte slides could be blocked preventing non-specific cell binding while satisfactory blocking was not obtained with the silanated or plain glass slides.

7. A type of leukemia cell may be identified using the claimed method by determining the differential pattern of interaction between discrete regions of immobilized immunoglobulins and cells in a biological sample, which is indicated by the relative interaction between each immunoglobulin and its cognate cell surface antigen. This pattern of interaction may result, for example, from differential density of cells that bind to a discrete spot on the array, the differential expression of particular antigens, and/or the number of antigens per cell (*see, e.g.,* specification, page 26, lines 11-20). The resulting pattern of interaction may be visualized as an image and/or quantified in another manner that indicates a differential signal, such as the +/- system, or the 8-bit greyness scale (1-256) used by the Medsaic Array Reader, illustrated in the present application *(see, e.g.,* specification, Tables 1-8).

8. Each of Valet et al., Terstappen et al., Verwer et al., and Orfao de Matos Correira E Val teaches flow cytometric methods for distinguishing different types of leukemias or lymphoid populations by detection of CD antigens expressed on a cell. By way of background, flow cytometry is the analysis of biological material by detection of the light-absorbing or fluorescing properties of cells passing in a narrow stream through a laser beam. These properties can depend on the auto-fluorescence of a cell population, or alternatively, fluorochromes can be bound to the cells that produce signals at different wavelengths. An optical absorbance or fluorescence profile of the sample is then produced. At the date of filing of the present application, typical flow cytometers could detect three fluorochromes concurrently on cells in a single sample.

9. I submit that a person having ordinary skill in the art at the time of filing USSN '959 would not have been motivated by any suggestion or teaching in Chang, or with any teaching regarding flow cytometry in Valet et al., Terstappen et al., Verwer et

al., and Orfao de Matos Correira E Val to achieve the claimed method. Chang teaches a general method for analyzing several antibody-antigen binding interactions but fails to provide any suggestion or motivation for using the method taught therein to construct an extensive array of immobilized antibodies for identifying a type of leukemia or any disease in a human subject.

10. Valet teaches a flow cytometry method that uses triple matrix classifiers, that is, groups of three antibodies specific for three different cellular antigens analyzed as a single parameter in three separate samples, to generate a triple matrix database. Thus, Valet fails to teach the claimed method that comprises concurrent analysis of the separate interactions between the immunoglobulins of the array and at least seven cell surface marker antigens. Valet lacks any suggestion that modification of the procedures disclosed therein is desirable; Valet teaches that a triple matrix pattern of antigen expression, even a single triple matrix pattern (*i.e.*, three antibodies specific for three different antigens), meets the criteria set forth for classification of several leukaemias and lymphomas (*see e.g,*. Valet, pages 286-287).

11. Terstappen fails to teach concurrent analysis of each immunoglobulin/antigen binding interaction and instead teaches a *sequential* analysis of *antibody pairs determined in sequential analyses by flow cytometry of at least 5 aliquots of a cell preparation taken from a patient.* Terstappen further teaches that the sequence of cell aliquot analysis and antibody pairs is important for practising the method disclosed therein (*see* Terstappen, column 2, lines 51-65). Thus, Terstappen teaches a method for classifying leukemias that uses different techniques and analyses and does not remotely suggest any desirability to combine the teachings therein with any other prior art to achieve the Applicants' invention.

12. Verwer fails to teach or suggest concurrent analysis of multiple individual antibody/antigen interactions and instead teaches multiple (8) sequential analyses for each blood or bone marrow sample of antibody/antigen pairs for fluorescence and light scatter determined by flow cytometry (*see* Verwer throughout). Moreover, Verwer

teaches a technique for specifically analyzing flow cytometry data, wherein the technique provides positional information of cell clusters that is matched across multiple aliquots of a sample (Verwer, column 3, lines 7-11). This method uses one of several statistical clustering algorithms preferably modified mutual nearest neighbour values. Thus, Verwer also fails to provide any teaching, suggestion, or motivation to combine or modify the teachings of the prior art to produce the Applicants' claimed method. Verwer teaches a method for classifying leukemias that uses different techniques and analyses and does not suggest any desirability to combine the teachings therein with any other prior art to achieve the Applicants' method for identifying a type of leukemia in a human.

13. Orfao de Matos Correira E Vale teaches a flow cytometry method for distinguishing the major types of human lymphocytes, T, B, and NK cells and subsets of these cell types, by expression of certain CD antigens. A mixture of 5 monoclonal antibodies conjugated with 3 different fluorochromes is used in 6 combinations on 6 aliquots of cells to determine by flow cytometry 12 lymphoid sub-populations. Expression of CD antigens as taught in Orfao de Matos Correira E Vale, such as CD3, CD4, and CD8, on lymphocytes and detection of these antigens with antibodies has been long known in the immunology art. Orfao de Matos Correira E Vale fails to teach or suggest that the CD antigen/antibody interactions disclosed therein may be applicable to any method for identifying a type of leukemia in a human subject. Orfao de Matos Correira E Vale teaches a method for identifying subsets of lymphocytes using a different technique and analysis than the claimed method and does not suggest any desirability to combine the teachings therein with any other prior art to achieve the Applicants' method for identifying a type of leukemia in a human.

14. With respect to the rejection of the claims over Hoeffler in view of Terstappen, I submit that a person having ordinary skill in the art at the time of filing USSN '959 would not have been motivated by any suggestion or teaching in either document to combine the teachings therein to achieve the claimed method. Hoeffler describes a method for screening very large numbers of uncharacterized antibodies for those specific for a given antigen on a protein. Thus, the antibodies of the array are unknown, rather than the antigens in the applied protein sample. Hoeffler also described

a microarray of uncharacterized antibodies used to compare expression profiles of cells. Note that in both these applications, proteins or lysates rather than intact cells are used, and the identities of the antibodies in the array are unknown. There is no discussion of a pattern of recognition for leukemia, or a disease signature. Rather, mention was made on P30 L28 of "a population of antibodies diagnostic for a variety of disorders on a single surface." The only example supported by data involving a microarray of known antibodies and a cell extract was example VII (P46). In this example, 8 antibodies were used to make an array that was used to detect a single protein, β-galactosidase, in an extract of CHO cells (Fig. 5). Hoeffler is silent regarding contacting immunoglobulin molecules of an array with a biological sample containing cells for detection of the interaction between the immunoglobulin and the cell surface antigen. Hoeffler as a whole teaches a method for detecting an antibody or an antigen, wherein the antigen may be purified or partially purified (Hoeffler, paragraph 42).

15. Terstappen fails to teach concurrent analysis of each immunoglobulin/antigen binding interaction and instead teaches a *sequential* analysis of *antibody pairs*. The patent teaches that according to the method described therein, the sequence of the cell aliquot analysis and antibody pairing is important (*see* Terstappen, column 2, lines 51-65).

16. None of the documents alone or in combination teach the presently claimed method. Furthermore, none of the documents indicates any desirability to modify either method disclosed therein to achieve the Applicants' claimed method. Hoeffler fails to suggest, teach, or motivate a person having ordinary skill in the art to combine its teachings with any other prior art teaching to obtain the claimed method for identifying a type of leukemia. Terstappen also fails to provide any teaching, suggestion, or motivation to combine or modify the teachings of the prior art to produce the Applicants' claimed method. Terstappen teaches a method for classifying leukemias that uses different techniques and analyses and does not remotely suggest any desirability to combine the teachings therein with any other prior art to achieve the Applicants' invention.

17. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and may jeopardize the validity of any patent issuing from this patent application.

R. I. Christopherson — 28 September 2004

**RICHARD IAN CHRISTOPHERSON** — Date

514614

# *Curriculum Vitae* of Professor Richard I. Christopherson

**School of Molecular and Microbial Biosciences**
**University of Sydney**
**Sydney**
**NSW 2006**

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## (a) Personal Details


| | |
|---|---|
| Address: | 137 Sutherland Street, Paddington, NSW 2021 |
| Telephone: | 02-9351-6031 (W), 02-9363-0802 (H), 0421 057 905 (M) |
| Fax: | 02-9351-4726 |
| Email: | ric@mmb.usyd.edu.au |


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|---|---|
| Born: | 7th August, 1949, at Melbourne, Victoria |
| Citizenship: | Australian |
| Married: | Wife, Julie; children, Sascha (27 years) and James (22 years). |
| Memberships: | Corresponding Member of the American Association for Cancer Research (AACR, 1995- )<br>Scientific Advisory Committee of the International Purine and Pyrimidine Society (2003- )<br>National Committee for Biochemistry of the Australian Academy of Science (1991-97).<br>Honorary Medical Officer, Royal North Shore Hospital (1995- )<br>Australian Society for Biochemistry and Molecular Biology (ASBMB)<br>Alternate Director and member of the Executive, Australian Proteome Analysis Facility (APAF)<br>Director, Biomedical Node of APAF (University of Sydney) |

## (b) Degrees

| | |
|---|---|
| 1970 | Bachelor of Science from the University of Melbourne majoring in Biochemistry and Chemistry. |
| 1973-76 (May) | Doctor of Philosophy (Biochemistry) from the University of Melbourne; thesis entitled "Interrelationships of Pyrimidine Biosynthesis in *Escherichia coli* K12". |

## (c) Appointments

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|---|---|
| 1971-72 | Tutor (full-time), Russell Grimwade School of Biochemistry, University of Melbourne. |
| 1976-78 (July) | Fellow of the Damon Runyon-Walter Winchell Cancer Fund, Biochemistry Department, School of Medicine, University of Southern California, Los Angeles, USA. |
| 1978-80 (June) | Special Fellow of the Leukemia Society of America, Biochemistry Department, School of Medicine, University of North Carolina at Chapel Hill, USA. |
| 1980-83 (January) | Research Fellow, Biochemistry Department, John Curtin School of Medical Research, Australian National University. |
| 1983-86 (January) | C.R. Roper Fellow in Medical Research, Russell Grimwade School of Biochemistry, University of Melbourne. |
| 1986-97 | Lecturer/Senior Lecturer/Associate Professor, Department of Biochemistry, University of Sydney. |

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| 1998-03 | Professor (Personal Chair) and Head, Department of Biochemistry/School of Molecular and Microbial Biosciences, University of Sydney. |
| 2004- | Professor (Personal Chair), School of Molecular and Microbial Biosciences, University of Sydney. |

## (d) Research

### (i) Grants Received (Total 1987-03: $9,210,457)

| | |
|---|---|
| **1987** | |
| NH&MRC | 33,461 |
| ARGS | 5,000 |
| Utah Foundation | 24,000 |
| University of Sydney Cancer Research Fund | 10,000 |
| Wellcome Australia Ltd. | 121,482 |
| | $193,943 |
| **1988** | |
| NH&MRC | 34,693 |
| NH&MRC | 24,833 |
| ARC | 6,000 |
| Wellcome Australia Ltd. | 122,960 |
| | $188,486 |
| **1989** | |
| NH&MRC | 35,810 |
| NH&MRC | 25,876 |
| ARC | 8,000 |
| Wellcome Australia Ltd. | 133,928 |
| | $203,614 |
| **1990** | |
| NH&MRC | 37,827 |
| ARC | 8,394 |
| Wellcome Australia Ltd. | 159,129 |
| University of Sydney Cancer Research Fund | 18,000 |
| Leo & Jenny Leukaemia & Cancer Foundation | 10,000 |
| NSW State Cancer Council (with Prof. B.D. Roufogalis) | 40,174 |
| | $273,524 |
| **1991** | |
| NH&MRC | 39,869 |
| University of Sydney Cancer Research Fund | 8,000 |
| USCRF (with Prof. B.D. Roufogalis) | 46,795 |
| Leo & Jenny Leukaemia & Cancer Foundation | 40,099 |
| University of Sydney major equipment grant | 30,000 |
| Department of Biochemistry equipment grant | 9,000 |
| | $173,763 |
| **1992** | |
| World Health Organization | 68,435 |
| NH&MRC | 92,092 |
| University of Sydney Cancer Research Fund | 42,252 |
| Parnell Laboratories (clinical trial analyses) | 26,100 |
| | $228,879 |
| **1993** | |
| NH&MRC | 95,849 |
| NH&MRC | 41,660 |
| Leo & Jenny Leukaemia & Cancer Foundation | 40,000 |
| World Health Organisation (US$52,137) | 75,742 |
| University of Sydney Cancer Research Fund | 42,252 |
| | $295,503 |
| **1994** | |
| NH&MRC | 96,904 |
| NH&MRC | 42,118 |
| World Health Organisation (US$65,137) | 98,692 |
| University of Sydney Cancer Research Fund | 42,252 |
| ARC small grant | 8,000 |
| | $287,966 |

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|---|---|
| **1995** | |
| NH&MRC | 42,792 |
| NH&MRC | 58,258 |
| WHO | 46,052 |
| ARC | 8,000 |
| | $155,102 |
| **1996** | |
| NH&MRC | 58,258 |
| University of Sydney infrastructure funds | 53,933 |
| University of Sydney Cancer Research Fund | 45,000 |
| ARC small grant | 8,000 |
| | $165,191 |
| **1997** | |
| NH&MRC | 61.951 |
| NH&MRC equipment grant (with C. Collyer & G. King) | 25,000 |
| ARC RIEFP grant (with C. Collyer) | 185,000 |
| University of Sydney Cancer Research Fund | 45,000 |
| Faculty of Medicine | 15,000 |
| | $331,951 |
| **1998** | |
| Wellcome Trust (Recombinant Protein Facility) | 172,388 |
| Faculty of Medicine | 10,000 |
| Ramaciotti Foundation (with P.W. Kuchel) | 25,000 |
| | $207,388 |
| **1999** | |
| Wellcome Trust | 172,388 |
| ARC Institutional Grant | 9,500 |
| Enterix Pty. Ltd. | 125,936 |
| | $307,824 |
| **2000** | |
| Enterix (with C.G. dos Remedios) | 300,469 |
| Wellcome Trust | 172,388 |
| ARC Small Grant | 23,000 |
| U2000 Fellowship (to M.A. Kamal) | 63,953 |
| | $559,810 |
| **2001** | |
| Wellcome Trust | 172,388 |
| U2000 Fellowship (to M.A. Kamal) | 63,953 |
| U2000 Fellowship (to R.I. Menz) | 31,976 |
| COMET grant (with C.G. dos Remedios, J. Chrisp) | 25,000 |
| | $293,317 |
| **2002** | |
| USyd Sesqui grant | 40,000 |
| AusIndustry Business Innovation Fund (with C dos Remedios, J Chrisp and B Hamdorf) | 500,000 |
| AusIndustry COMET grant (with C dos Remedios, J Chrisp and B Hamdorf) | 25,000 |
| Wellcome Trust Major Equipment Grant | 14,000 |
| USyd U2000 Fellowship (to MA Kamal) | 63,953 |
| | $642,953 |
| **2003** | |
| USyd Sesqui, Equipment (with RC Baxter, R Christopherson and N King) | 208,000 |
| AusIndustry, Major National Research Facility, Australian Proteome Analysis Facility (with P Bergquist *et al.*) | 1,790,000 |
| NH&MRC | 80,000 |
| Medsaic (spin-off company, CEO J Chrisp) | 239,421 |
| Medsaic | 83,821 |
| Medsaic | 259,730 |
| USyd U2000 Fellowship (to MA Kamal) | 31,976 |
| | $2,692,948 |

**2004**

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|---|---|
| USyd Sesqui, Equipment (with M. Crossley, K. Downard, R. Overall, W. Britton, J Triccas, N Jacques, A. Weiss) | 70,000 |
| NH&MRC | 70,000 |
| ARC (with R Baxter) | 90,000 |
| ARC LIEF for MALDI-TOF/TOF mass spectrometer (with J. Gorman, at UQ) | 1,649,750 |
| Medsaic (spin-off company) | 128,545 |
| | $2,008,295 |

### (ii) Research Group Members

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|---|---|
| Larissa Belov PhD | Research Fellow (with Medsaic at ATP) |
| Ben Crossett PhD | Research Fellow (Manager, Proteomics Unit) |
| Pauline Huang MSc | Research Assistant (with Medsaic at ATP) |
| Camilla Chan BSc(Hons) | Research Assistant |
| Nicole Barber BSc(Hons) | PhD student |
| Carlos Cassano BSc(Hons) | PhD student |
| Maryam Shojaei BSc(Hons) | PhD student |
| Louise Bransgrove BSc(Hons) | PhD student |
| Silke Henrich | PhD student |
| Daniel Morgan | BSc(Hons) student |
| Stephen P Mulligan PhD MBBS FRACP | Adjunct Senior Lecturer |
| Stephen D Lyons PhD MBBS MRACOG | Adjunct Lecturer |

### (iii) Research Projects

1. *Leukaemia membrane proteomics.* Procedures have been developed for sub-cellular fractionation of leukocytes. Plasma membrane and nuclear proteomes will be determined for leukaemia cell lines treated with various drugs, and leukaemia cells from patients.

2. *Immunophenotyping solid tumours.* Biopsies of colon cancer have been reduced to single cell suspensions and a procedure has been developed for getting the cells to re-express intact surface molecules. An antribody microarray is under development for immunophenotyping cells from colon cancer , initially using human cell lines.

3. *Direct immunophenotyping of whole blood lysates.* The current procedure used for immunophenotyping leukocytes from blood samples is to purify them by Histopaque centrifugation which also removes a major proportion of the predominant neutrophils. The leukocytes are then specifically captured on a CD antibody microarray. Leukocytes can be analyzed directly by flow cytometry following selective lysis of erythrocytes. We propose to develop a protocol which enables blood lysates to be analyzed using the CD antibody microarray in the presence of the predominant neutrophils.

4. *Detection of intracellular antigens in captured cells.* Leukocytes captured on a CD antibody microarray will be permeabilised and probed with soluble, fluorescently-labelled antibodies against intracellular protein markers such as p210, which is associated with chronic myeloid leukaemia (CML) and acute lymphocytic leukaemia (ALL). p210 is a tyrosine kinase that is inhibited by the novel anticancer drug, Glivec.

5. *A database for leukaemias and lymphomas.* Statistical analysis of sub-types (e.g. AML) using principal component plots will be prepared from the more than 300 immunophenotypes already determined for a variety of leukaemias and lymphomas from patients. Such plots show segregation of disease types in component space and should show that an extensive immunophenotype provides sufficient information for diagnoses.

6. Development of recombinant antibodies. The CD antibody microarray will detect unusual pairs of antigens expressed on particular leukaemias. Antibody-like molecules have been designed that will only kill cells expressing an unusual pair of CD antigens.

7. *Cloning and expression of malarial pyrimidine pathway enzymes.* In collaboration with Dr Ian Menz, now at Flinders University, we have cloned the malarial genes encoding dihydroorotase, orotate phosphoribosyltransferase, and OMP decarboxylase. We have obtained cloned genes for carbamyl phosphate synthetase, aspartate transcarbamylase and CTP synthetase from collaborators at the University of NSW. We are over-expressing and purifying these enzymes, and growing protein crystals for determination of their three-dimensional structures.

8. *The catalytic mechanisms of dihydroorotases.* Enzymes from hamster, *P. falciparum*, *Bacillus caldolyticus* and *Escherichia coli.* These enzymes have been cloned, over-expressed and purified, and comparative kinetic analyses are underway. We are also characterizing Type 1 and 2 dihydroorotases which may have one and two zinc atoms, respectively, at their active sites.

**(iv) Key Research Discoveries**

1. *Expression of hamster dihydroorotase.* The central DHOase domain of the trifunctional protein, DHO synthetase, has been cloned, sequenced, expressed in *E. coli*, purified in hundreds of milligrams and crystallised. We have published a low resolution X-ray structure.

2. *The catalytic mechanism of dihydroorotase.* We have shown there is a zinc atom at the active site coordinated by 3 histidine residues which participates in catalysis. Site-directed mutagenesis and kinetic experiments have enabled elucidation of the catalytic mechanism of DHOase.

3. *Inhibitors of dihydroorotase.* A series of potent inhibitors of DHOase has been rationally designed from a knowledge of the catalytic mechanism of the enzyme. TDHO ($K_i$ = 0.85 μM) may be regarded as a chelating inhibitor, while HDDP ($K_i$ = 0.74 μM) and OAPC ($K_i$ = 7.4 μM) are transition-state analogues. Alkyl esters of TDHO and HDDP induce inhibition of DHOase and hence *de novo* pyrimidine biosynthesis in leukaemia cells and malaria growing in culture with $IC_{50}$ values of less than 20 μM.

4. *The mechanism of the anti-purine effect of methotrexate.* We have found that the high levels of dihydrofolate polyglutamates induced by methotrexate in leukaemia cells inhibit amido phosphoribosyltransferase catalysing the first step of the *de novo* purine pathway. Dihydrofolate polyglutamates and some other folate derivatives such as piritrexim bind at a new inhibitory allosteric site on this enzyme and induce formation of an inactive 7.2 S dimer rather than the inactive 10.2 S tetramer induced by purine nucleoside monophosphates. Elucidation of the true antipurine effect of methotrexate is very important because this antifolate is used to treat leukaemia, breast cancer, rheumatoid arthritis and lupus.

Preliminary data suggest that the antifolate, Lometrexol, which is known to inhibit the third reaction of the *de novo* purine pathway, also inhibits amido phosphoribosyltransferase (reaction 1) which would be the primary blockade of purine biosynthesis.

5. *Measurement of 2'-deoxynucleoside-5'-triphosphates in malaria and fresh human leukaemia cells.* We have developed HPLC procedures which enable the direct measurement of dNTPs in cells taken from patients with Chronic Lymphocytic Leukaemia (CLL) enabling a detailed investigation of the mechanisms of action of the drugs cladribine, fludarabine and pentostatin. These techniques have also been used to measure dNTPs in the malarial parasite, *Plasmodium falciparum,* growing in erythrocytic culture. We have found that the antifolate, WR99210, inhibits dihydrofolate reductase in the parasite but there is an additional site of action which remains to be determined. Parasites exposed to a variety of drugs and orotate maintain relatively constant levels of dCTP suggesting that this dNTP is compartmentalised or that its levels are maintained via unknown regulatory mechanisms.

6. *AICAR transformylase-IMP cyclohydrolase.* The bifunctional enzyme AICAR transformylase-IMP cyclohydrolase has been purified to homogeneity from human leukaemia cells. A purine nucleoside monophosphate analogue (MIMP) has been synthesised which is a potent inhibitor of IMP cyclohydrolase ($K_i$ = 94 nM).

7. *A CD antibody microarray.* This microarray of immobilized antibodies against surface molecules found on cells provides extensive immunophenotypes of leukocytes and cells from solid tissues. Using this novel technique, consensus immunophenotypes have been established for the common leukaemias. We have proposed that an extensive immunophenotype should be sufficient to diagnose leukaemias without using additional criteria of cell morphology, cytochemistry and cytogenetics. A scanner and software have been developed by a spin-off company, Medsaic, and a diagnostic kit for leukaemias will be marketed in late 2003.

**(v) Collaborators**

1. Immunophenotyping leukaemias from patients using a CD antibody microarray. Dr. S.P. Mulligan, Department of Haematology, Concord Hospital.

2. Proteomic analysis of breast cancer cells undergoing apoptosis. Prof R Baxter, Kolling Institute of Medical Research, Royal North Shore Hospital, Sydney.

3. Analysis of subpopulations of normal germinal center B cells, memory and naive B cells using a CD antibody microarray. Prof. Carl A.K. Borrebaeck, Department of Immunotechnology, Lund University, Sweden.

4. Cloning, expression, purification, crystallization and structural determination of enzymes from the *de novo* pyrimidine pathway in the malarial parasite, *P. falciparum.* Dr R Ian Menz, School of Biological Sciences, Flinders University.

5. Protein crystallography with recombinant hamster and bacterial dihydroorotases and malarial OMP decarboxylase. Drs M. Maher, D. Langley and M. Guss, School of Molecular and Microbial Biosciences, University of Sydney.

6. Membrane proteomics of leukaemia cell lines and leukaemias from patients. Prof Mark Baker and Dr Stuart Cordwell, Australian Proteome Analysis Facility, Macquarie University.

## (e) Publications

### (i) Articles in Refereed Journals


1. Christopherson, R.I. & Finch, L.R. (1976) Anal. Biochem. 73, 342-349. A radioisotopic method for the assay of aspartate carbamoyltransferase and carbamoyl phosphate.
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## (ii) Manuscripts Submitted (copies available)

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87. Woolfson A, Tom B, Stoner K, Stebbing J, Kreil D, Gilks W, Mulligan S, Wildfire A, Erber W, Gazzard B, Christopherson R, Scott M (2004) Science *Brevia submitted.* Conservation of a unique CD cell surface mosaic in clinically heterogeneous HIV infected individuals
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## (iii) Manuscript in Preparation (draft copies available)

**89.** Huang DTC, Kaplan J, Menz RI, Katis VL, Wake RG, Anderson M, Cleland WW, Christopherson RI (2004) Biochemistry. Catalysis by mesophilic and thermophilic dihydroorotases: Effects of temperature, inhibition and denaturation.
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## (iv) Chapters in Books

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## (v) Patents

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113. Christopherson, R.I., Mackay, J.P., Matthews, J. (2002) Australian Provisional Patent filed 28/11/02. Therapeutic agents.
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## (vi) Recent Invited International Lectures

- *Immunophenotyping of leukaemias using a cluster of differentiation antibody microarray*, IBC meeting, Hamburg, September 2002.
- *Immunophenotyping of leukaemias using a cluster of differentiation antibody microarray*, IBC 13th International Conference on Antibody Engineering, San Diego, December 2002.
- *Consensus immunophenotypes for the common leukaemias using a CD antibody microarray*, IBC 4th Annual Protein Microarrays, San Diego, April 2003.
- *Cloning and expression of malarial pyrimidine enzymes*, Joint 11th International and 9th European Symposium on "Purines and Pyrimidines in Man", Egmond aan Zee, the Netherlands, June 2003.

- *Channelling of intermediates in the biosynthesis of pyrimidines, purines and urea.* The Mary Ellen Jones Lecture at the International Conference on Arginine and Pyrimidines (ICAP2004), Marburg, Germany, September 2004.
- *New technology for diagnosing leukemias.* Department of Pharmacology, University of North Carolina, Chapel Hill, USA, October 2004.

**(f) Teaching Innovations**

- I was heavily involved in the development of the Graduate Medical Program (USydMP) about 5 years ago. I wrote many documents for this novel course based upon Problem Based Learning, and was the pre-clinical coordinator for the first case "Mr Sarich's Chest Pain", written with the Professor of Cardiology at Royal Prince Alfred Hospital, Prof. Phil Harris. The USydMP is web-based and students view details of the case of the week on computers in tutorial rooms. This first case was presented to the Australian Medical Council for accreditation of the course and is available to the public as "a sample week" of the course at

  http://www.gmp.usyd.edu.au/vguide/students/samplew/mscp_fset.html

- I have developed a new course "The Biochemistry of Cancer" for Biochemistry 3 students which covers current aspects of epidemiology, causes, molecular mechanisms and treatment. This course of 8 lectures has largely been developed from published work, some of the information is too recent to be found in text books.

- I have developed a Biochemistry 3 practical class experiment "Adenosine Deaminase Deficiency in Human Lymphocytes" which is run over 4 days. This experiment uses high pressure liquid chromatography (HPLC) with computerized data acquisition and processing. The emphasis is on using sophisticated equipment and software to link basic biochemical properties of cells with a clinical disorder.

**(g) Administration, Service to the Profession and Community**

- Foundation Head, School of Molecular and Microbial Biosciences University of Sydney (1998-03)
- Chair, Faculty Promotion Committee to Lecturer and Senior Lecturer, Faculty of Medicine (2000-02)
- National Committee for Biochemistry of the Australian Academy of Science (1991-97)
- Referee panel for the National Health & Medical Research Council
- Grants Evaluation Panel of the Australian State Cancer Councils
- Convenor and organizer, National Heads of Schools (Biochemistry) meeting, University of Sydney, September 2003
- Panel of reviewers for *Biochemistry, Biochemical Pharmacology* and *International Journal of Biochemistry and Cell Biology*

**(h) Referees**

1. Prof. F.W.E. Gibson F.R.S. F.A.A.
   John Curtin School of Medical Research
   Australian National University
   P.O. Box 334
   Canberra, A.C.T. 2601
   Email: frank.gibson@anu.edu.au

2. Prof. W.W. Cleland
   (Member of the National Academy of Sciences)
   Institute for Enzyme Research
   University of Wisconsin-Madison
   1710 University Avenue
   Madison, Wisconsin 53705-4098
   USA
   Email: cleland@enzyme.wisc.edu

3. Prof. R.G. Wake F.A.A
   Department of Biochemistry
   University of Sydney
   Sydney,
   NSW 2006
   Email: g.wake@mmb.usyd.edu.au

4. Prof. W.H. Sawyer
   Department of Biochemistry
   University of Melbourne
   Parkville, Vic., 3052
   Email: wsawyer@netspace.net.au

5. Prof. B.D. Roufogalis
   Department of Pharmacy
   University of Sydney
   Sydney, NSW, 2006
   Fax: 02-9351-4447
   Email: basilr@pharm.usyd.edu.au